Minichromosome maintenance protein 10 as a marker for proliferation and prognosis in lung cancer

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Abstract. DNA replication is a vital process in cell division where anomalies can lead to tumorigenesis. Minichromosome maintenance complex component 10 (MCM10) plays a crucial role in this process. However, the role of MCM10 in lung cancer pathogenesis remains to be elucidated. In current study, using the publicly available lung cancer Gene Expression Omnibus (GEO) datasets, and Oncomine and the Cancer Genome Atlas databases, an increased expression of MCM10 was found in lung cancer tissues compared to normal lung tissues. The high expression of MCM10 was subsequently validated in clinical specimens by reverse transcription-quantitative PCR and immunohistochemistry. Analysis of the GEO datasets revealed that the high MCM10 expression was significantly associated with early and late recurrence, pathological stage and worse overall survival (OS). Cox's proportional hazards regression analyses revealed that MCM10 expression was an independent risk factor for poor OS and worse recurrence-free survival both in univariate and multivariate analysis. Furthermore, the increased expression of MCM10 was enriched in cell cycle-related processes, while *in vitro* transfection with small interfering RNA targeting MCM10 significantly suppressed cell viability, clone formation and induced G1 phase arrest in A549 and H661 cell lines by regulating the expression of cyclin D1 (CCND1). In addition, the current results indicated a combined effect of MCM10-CCND1 in predicting the prognosis of lung cancer patients. Altogether, the present study provided a novel potential molecular mechanism of lung cancer progression and may aid in development of novel treatment strategies.

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Introduction

Lung cancer is the leading cause of cancer-related deaths (1). The recent statistics by the American Cancer Society showed that the mortality and morbidity due to lung cancer in 2018 was approximately 154,050 and 234,040, respectively (2). Despite the advances in surgical techniques and molecular therapy for lung cancer, the 5-year survival of patients with non-small cell lung cancer (NSCLC) is only 21% (3). Based on the Surveillance, Epidemiology, and End Results data report in 2014, the rate of lung cancer diagnosis in early stages is only 16% (3). Moreover, 40 and 60% patients with stages IB and II lung cancer, respectively, relapse despite receiving surgery and adjuvant chemotherapy (4). Identification of new biomarkers to assess the prognosis of lung cancer is crucial for effective clinical decision-making and survival improvement.

Minichromosome maintenance complex component 10 (MCM10) is a conserved component of an eukaryotic replication fork (5,6) and originally identified as an allele of DNA43 in *Saccharomyces cerevisiae* (7,8). Along with other DNA replication proteins, it plays a vital role in DNA replication-related processes such as promoting the initiation of DNA replication (9), activating helicase (10-12) and contributing to polymerase loading (13,14), as well as controlling replication fork stability to sustain elongation (15,16). However, the expression level of MCM10 is abnormally increased during tumorigenesis (17-19). It has been demonstrated that overexpression of MCM10 is driven by the EWS RNA binding protein 1 (EWS)/Fli-1 proto-oncogene, ETS transcription factor (FLI1)-nuclear receptor subfamily 0 group B member 1 (DAX1) interaction in Ewing's sarcoma (17). In addition, in a previous study, MCM10 was significantly elevated in medulloblastoma cell lines and tissues when compared with corresponding control groups, and knockdown of MCM10 inhibited the proliferation of medulloblastoma cells (18). Another study revealed that high MCM10 expression was

observed in glioma samples and related to tumor grade (19). Furthermore, abnormally increased MCM10 expression was found to be associated with malignant clinicopathological characteristics, worse disease-specific survival and inferior metastasis-free survival in urothelial carcinoma (20).

Despite the elevated MCM10 levels found in various cancer types, its role in lung cancer pathogenesis remains elusive. Therefore, further research is required to uncover the molecular mechanism and prognostic implication of MCM10 in lung cancer. To this end, MCM10 expression levels in lung cancer versus non-tumor tissue, the correlation between MCM10 expression and clinicopathologic characteristics, and the prognostic potential of MCM10 expression in lung cancer were investigated. Furthermore, in the current study, the expression of MCM10 was knocked down by small interfering RNA to explore its biological role and molecular mechanism involved in the pathogenesis of lung cancer.

Materials and methods

Cell culture and transfection. Lung cancer cell lines A549 and H661 were obtained from the American Type Culture Collection and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml of streptomycin, and maintained in 5% CO_2 atmosphere at 37˚C. Small interfering RNA (siRNA) sense, 5'-GCACAAACTTGATCATCCA-3' targeting the human MCM10 (siMCM10) and negative control siRNA (NC; cat. no. NControl_05815) synthesized by Guangzhou RiboBio Co., Ltd. 100 nM siRNA and NC were transiently transfected into lung cancer cells using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cells were used for subsequent experimentation after 48-h transfection.

Clinical specimens. A total of 38 pairs of primary lung cancer and adjacent normal tissue samples were obtained from patients who underwent surgical operations at the Renmin Hospital of Wuhan University between May 2017 and November 2017 (age range, 41-75 years; 23 males and 15 females). All patients provided informed consent. Samples were immediately frozen in liquid nitrogen for MCM10 mRNA expression analysis. Human lung tissue microarrays including 56 pairs of primary lung cancer and corresponding adjacent normal lung/bronchiole tissues were purchased from Shanghai Outdo Biotech Company Co., Ltd. The tissue microarrays contained 24 adenocarcinoma (ADC), 13 squamous cell carcinoma (SCC), 7 adenosquamous carcinoma (ASC), 5 large cell lung carcinoma (LCLC) and 7 of small-cell lungs carcinoma (SCLC) samples.

Bioinformatics and statistical analysis. MCM10 transcript level in lung cancer was determined from the Oncomine database (https://www.oncomine.org). A total of 7 datasets were selected using the following filter settings: MCM10, analysis type: Cancer vs. Normal analysis; Cancer type: Lung cancer. Comparison of MCM10 across 9 analyses was performed based on the screening criteria. The details of the analyses are listed in Table I. Gene expression omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) was used as a source to downloaded GSE30219 (21), GSE19188 (22) and GSE31210 (23) microarrays datasets. A total of 22 cases in GSE31210 were excluded from univariate and multivariate Cox proportional hazards regression analysis due to incomplete resection or adjuvant therapy. Tumor recurrence was classified as either early or late using 2 years as the cutoff. The expression pattern of MCM10 and the corresponding clinical data were extracted and analyzed. Kaplan-Meier (KM) Plotter (http://kmplot.com/analysis/index.php?p=service&cancer=lung) was used to determine the prognostic significance of MCM10. The following settings were used to obtain the KM survival plots: Gene symbol, MCM10; split patients by, median; survival, overall survival (OS), follow up threshold, all; histology, all/ADC/SCC; all other settings, all. The categorization of MCM10 expression (low vs. high) was based on the median expression level. Log rank P-values and hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated and extracted from the KM Plotter webpage. The expression level of MCM10 in the Cancer Genome Atlas (TCGA) dataset was also analysed by UALCAN (http://ualcan.path.uab.edu). The role of MCM10 in biological pathways was investigated using gene set enrichment analysis (GSEA; version 3.0; Broad Institute, Inc.) and the GSE3141 (24) dataset with functional gene set files (GSEA file, c2.cp.v4.0.symbols.gmt) to access enriched gene sets.

Immunohistochemistry (IHC) assay. Paraffin was removed from the microarray tissue samples by treating with xylene twice for 10 min followed by rehydration with gradient alcohol for 5 min. Sodium citrate buffer (Wuhan Servicebio Technology Co., Ltd.; 10 mM; pH 6) was used for antigen retrieval and the samples were heated at 100˚C in a microwave oven. Endogenous peroxidase activity was inhibited by treating the slides with 3% hydrogen peroxide. The tissue microarray was incubated with primary antibodies for MCM10 at 4˚C (1:100; cat. no. DF12162; Affinity Biosciences). After overnight incubation, the microarray slides were rinsed with PBS and incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Wuhan Servicebio Technology Co., Ltd.; 1:200; cat. no. GB23303) at 37˚C for 30 min and the reaction was visualized by using a fresh solution of 3,3'-diaminobenzidine. The same criteria were used for evaluate the staining intensity and proportion of positive stained cells in each sample. The staining intensity was scored as 0 (no staining), 1 (pale yellow), 2 (yellow) and 3 (brown). The proportion of stained cells was defined as 0 (0-5% positive staining), 1 (6-25% positive staining), 2 (26-50% positive staining), 3 (51-75% positive staining) and 4 (>75% positive staining). The multiplication of staining intensity score and proportion of positive cells score was used as the IHC index. The IHC index ≥5 was defined as high expression of MCM10, while IHC index ≤4 was defined as MCM10 low expression.

Western blotting. After washing with PBS, total protein was extracted from transfected cells using RIPA (Beyotime Institute of Biotechnology) supplemented with a protease inhibitor cocktail PMSF (Kangchen BioTech Co., Ltd.) on ice for 30 min followed by quantification of the protein in the lysate using a BCA kit purchased from Thermo Fisher Scientific, Inc.

Table I. Comparison of minichromosome maintenance complex component 10 expression across nine analyses.

Legend	Author, year	Groups compared	Fold-change	P-value	Sample size (Gene Rank)	Refs.
1	Hou *et al*, 2010	LCC vs. normal	6.446	$7.96x10^{-8}$	328 (5%)	(22)
2		ADC vs. normal	2.894	$1.28x10^{-12}$	335 (5%)	
3		SCC vs. normal	4.099	$4.06x10^{-16}$	196 (5%)	
4	Landi *et al*, 2008	ADC vs. normal	1.509	$1.22x10^{-9}$	1,013 (10%)	(45)
5	Okayama *et al*, 2012	ADC vs. normal	1.792	$1.92x10^{-9}$	1,584 (10%)	(23)
6	Stearman *et al*, 2005	ADC vs. normal	1.733	$5.36x10^{-14}$	659 (5%)	(46)
7	Su *et al*, 2007	ADC vs. normal	2.026	$9.00x10^{-3}$	2,144 (25%)	(47)
8	TCGA	PADC vs. normal	1.098	$7.60x10^{-2}$	3,318 (25%)	
9	Wachi *et al*, 2005	SCC vs. normal	1.513	$8.00x10^{-3}$	1,165 (10%)	(48)

ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; PADC, papillary lung adenocarcinoma. Legend corresponds with the analyses presented in Fig. 1D. The rank for MCM10 can be automatically accessed based on the screening criteria.

Protein samples were separated using SDS-PAGE (10% gel) and transferred onto a polyvinylidene difluoride membrane at 200 mA for 2 h. Following an overnight incubation with an anti-MCM10 antibody (1:3,000; Abcam; cat. no. ab3733) or an anti-cyclin D1 (CCND1) antibody (1:3,000; ABclonal Biotech Co., Ltd.; cat. no. A10757) at 4˚C, the membranes were incubated with a goat anti-rabbit IgG (1:10,000; ProteinTech Group, Inc., cat. no. 10285-1-AP) at 37˚C for 1 h after washing with TBS with Tween-20. The protein signal was detected using an Novex™ ECL Chemiluminescent Substrate Reagent kit (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the cell lines and tissues were isolated using the TRIzol® reagent (Takara Bio, Inc.) and quantified with NanoDrop™ 2000 (Thermo Fisher Scientific, Inc.). Using the PrimeScript RT-PCR kit (Takara Bio, Inc.), 2 μg RNA was converted to cDNA following the manufacturer's protocol. The quantification of gene expression was performed by qPCR using SYBR Premix Ex Taq™ (Takara Bio, Inc.). The Cq value of target gene was normalized according to the reference gene (β-actin) using the $2^{-\Delta\Delta Cq}$ method (25). The following thermocycling conditions were used: 50˚C for 30 min, 94.5˚C for 15 min; 40 cycles of 96˚C for 30 sec and 59.7˚C for 1 min. The primer sequences used for the qPCR analysis were as follows: MCM10 forward, 5'-CCCCTACAGACGATTTCT CGG-3' and reverse, 5'-CAGATGGGTTGAGTCGTTTCC-3'; CCND1 forward, 5'-GCTGCGAAGTGGAAACCATC-3' and reverse, 5'-CCTCCTTCTGCACACATTTGAA-3'; β-actin forward, 5'-GAAGAGCTACGAGCTGCCTGA-3' and reverse, 5'-CAGACAGCACTGTGTTGGCG-3'; CDK4 forward, 5'-AT GGCTACAAGCAGATATGAG-3' and reverse, 5'-TCACTC GGGGTTGCCCTC-3'. CCNB1 forward; 5'-AATAAGGCG AAGATCAACATGGC-3' and reverse, 5'-TTTGTTACCAAT GTCCCCAAGAG-3'; P27 forward, 5'-AGGAGATGTAAC TATCGGACCTC-3', and reverse, 5'-GTTCCCTTCGCCAAT CACTATT-3'; p21 forward, 5'-TCCTGGAGCAGACCACCC CG-3' and reverse, 5'-GGGGTGGGACAGGCACCTCA-3'; P19 forward, 5'-GGGTTTTCGTGGTTCACATCC-3' and reverse, 5'-CTAGACGCTGGCTCCTCAGTA-3'; CCNA2 forward, 5'-GGATGGTAGTTTTGAGTCACCAC-3' and reverse, 5'-CACGAGGATAGCTCTCATACTGT-3'; CCNE1 forward, 5'-GCCAGCCTTGGGACAATAATG-3' and reverse, 5'-CTTGCACGTTGAGTTTGGGT-3'; CDK6 forward, 5'-TC TTCATTCACACCGAGTAGTGC-3' and reverse, 5'-TGAGGT TAGAGCCATCTGGAAA-3'; CDK2 forward, 5'-CCAGGA GTTACTTCTATGCCTGA-3' and reverse, 5'-TTCATCCAG GGGAGGTACAAC-3'; CDK1 forward, 5'-GGATGTGCTTAT GCAGGATTCC-3' and reverse, 5'-CATGTACTGACCAGG AGGGATAG-3'.

Cell Counting Kit-8 (CCK-8) and colony formation assays. CCK-8 (Dojindo, Molecular Technologies, Inc.) and colony formation assays were used to determine the proliferation ability of lung cancer cells. For the CCK-8 assay, 3,000 cells were seeded in 96-well cell culture plates and allowed to grow overnight. The cells were then transfected with siRNA as aforementioned for 48 h. At indicated culture points (0, 24 and 48 h), 10 μl CCK-8 was added to each well and incubated for another 2 h at 37˚C. The measurement of absorbance was done at a wavelength of 450 nm. For the colony formation assay, after 48 h of transfection, the cells were seeded in six-well cell culture plates at a density of 500 cells/well for 14 days. The number of colonies was counted after fixing and staining with 1% crystal violet (containing with 4% methanol) for 10 min at 37˚C.

Cell cycle and apoptosis analysis. Cell cycle progression was assessed using cell cycle detection kit (KeyGEN BioTech Co., Ltd.; cat. no. KGA511) 48 h post transfection. Cells were resuspended in 70% ethanol at -20˚C overnight. The cells were washed with cold PBS, subsequently treatment with 100 μl RNase for 30 min at 37˚C and 400 μl PI for 30 min at 4˚C before flow cytometry analysis. The DNA content was analyzed with ModFit software (version 3.2; Verity Software House). Cell apoptosis was detected by flow cytometry (BD FACSARia™ III; BD Biosciences) after staining with Annexin V-allophycocyanin and PI (BD Biosciences; cat. no. 556547) according to the manufacturer's protocol. The apoptosis rate was calculated as the sum of early and late apoptosis.

Statistical analysis. GraphPad Prism (version 5.0; GraphPad Software, Inc.) and SPSS (version 17.0; SPSS, Inc.) were used for

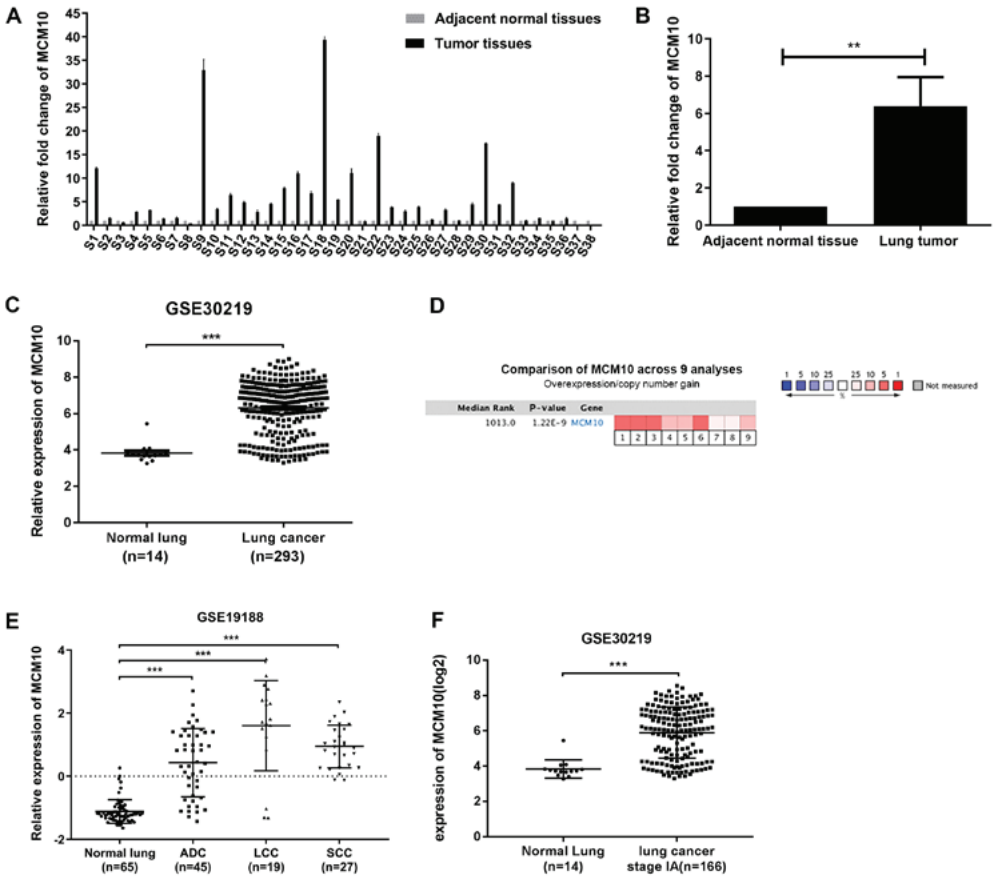

Figure 1. Expression of MCM10 is significantly elevated in lung cancer. (A) Reverse transcription-quantitative PCR analysis of MCM10 mRNA expression in 38 lung cancer tissues and adjacent normal tissues. (B) Comparison of the expression levels of MCM10 in lung cancer and adjacent tissues. (C) Comparison of the expression level of MCM10 in lung cancer tissues and normal lung tissues in from the GSE30219 dataset. (D) Cluster analysis of MCM10 expression in different lung cancer subtypes using data from the Oncomine database. Red, significant overexpression. Different shades of red indicate different gene ranks. (E) Analysis of MCM10 expression in normal lung and different histological subtypes of lung cancer in the GSE19188 dataset. (F) Analysis of MCM10 expression in normal lung and stage IA lung cancer tissue using data from the GSE31210 dataset. Data are presented as the mean ± SEM, n=3. **P<0.01 and ***P<0.001. MCM10, minichromosome maintenance complex component 10; ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; S, sample.

statistical analysis. Differences between paired samples were analyzed using a paired Student's t-test. Independent groups were analyzed using an unpaired Student's t-test. Multiple groups were compared using one-way ANOVA followed by Dunnett's test. The association between the expression level of MCM10 and the clinicopathological factors was explored using the Chi-square test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. Factors with prognostic significance in the univariate analysis were included in the subsequent multivariate analysis. The diagnostic value of MCM10 was evaluated by receiver operating characteristic (ROC) analysis and area under the curve (AUC). Data are presented as the mean ± SEM of three independent experiments, each performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of MCM10 in lung cancer. Quantification of the MCM10 expression level in 38 paired lung cancer and adjacent normal tissue samples was performed (Fig. 1A). The results revealed a significantly elevated MCM10 mRNA expression level in lung cancer tissues compared with the normal tissues (Fig. 1B). Subsequently, the GSE30219 dataset was selected to further assess the differences in MCM10 expression between cancerous and normal lung tissue samples. The results were consistent with the data derived from patients recruited in the present study (Fig. 1C). The expression analysis of MCM10 in different histological subtypes was carried out using the Oncomine database and GSE19188 dataset. The Oncomine results demonstrated the overexpression of MCM10 in lung tissues across 9 analyses. The median rank of MCM10 was 1,013 among upregulated genes by cluster analysis in lung cancer (Fig. 1D and Table I). MCM10 expression was significantly higher in ADC, SCC and SCLC compared with normal lung tissues (Fig. 1E). The expression level of MCM10 was also significantly upregulated in lung cancer compared with normal samples included in TCGA database (Fig. S1). Moreover, stage IA of lung cancer exhibited a higher MCM10 expression than normal lung tissue (Fig. 1F). In order to evaluate the diagnostic value of MCM10, the GSE30219 dataset was used to perform ROC analysis. The result showed that MCM10 discriminated a lung cancer patient from a healthy individual with a ROC area under the curve of 0.927 (95% CI, 0.0.887-0.966) (Fig. S2).

Table II. Association between MCM10 expression and clinicopathological features of 204 stage I-II primary lung adenocarcinoma patients from the GSE31210 dataset.

Characteristic	No. of patient	MCM10 expression		Chi-square value	P-value
		High	Low		
Age, years				1.59	0.207
≤60	99	54	45		
>60	105	48	57		
Sex				1.59	0.207
Male	95	52	43		
Female	109	50	59		
Pathological Stage				19.467	<0.001
IA	109	42	67		
IB	53	27	26		
II	42	33	9		
Early recurrence				16.071	<0.001
Yes	30	24	6		
No	150	60	90		
Late recurrence				10.248	0.001
Yes	24	18	6		
No	150	60	90		

Using 2 years as the cutoff, tumor recurrence was classified as either early or late. Patient samples were divided into high and low expression groups based on the median expression of MCM10. MCM10, minichromosome maintenance complex component 10.

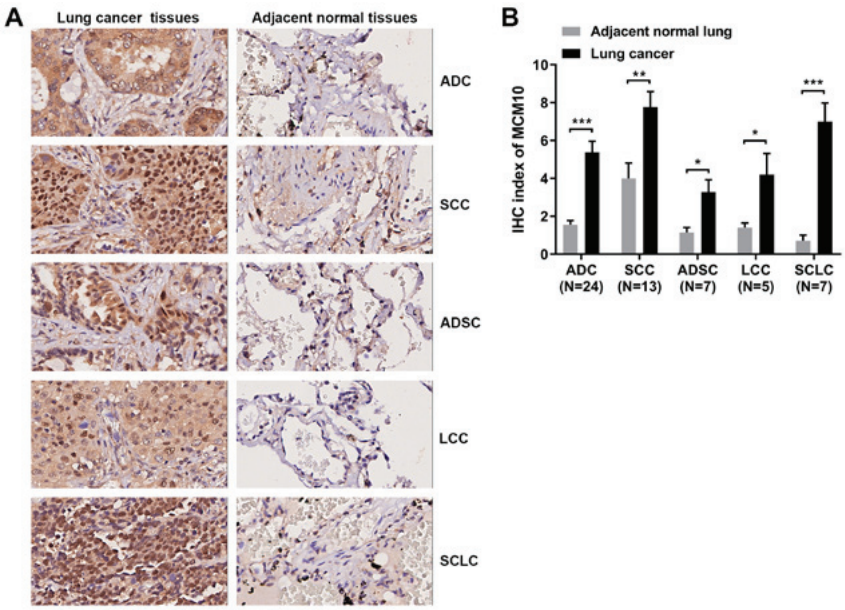

Figure 2. IHC analysis. (A) Representative images of MCM10 expression in different histological subtypes of lung cancer tissues compared with corresponding normal tissue (magnification, x40). (B) Comparison of the expression levels of MCM10 in different lung cancer subtypes and adjacent tissues using the IHC index. *P<0.05, **P<0.01 and ***P<0.001. MCM10, minichromosome maintenance complex component 10; ADC, adenocarcinoma; ADSC, adenosquamous carcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; SCLC, small-cell lung cancer; IHC, immunohistochemical staining.

Furthermore, upon investigation of the MCM10 expression level in 56 pairs of cancer and adjacent normal tissue samples from the lung tissue microarray by IHC, the positive staining of MCM10 was mainly found in the nucleus and partly in the cytoplasm of the lung cancer cells (Fig. 2A). Among the 56 cases of lung cancer, 33 (58.9%) lung cancer tissues showed high expression (IHC index ≥5) of MCM10, whereas the adjacent normal lung/bronchiole tissues had a low level or negative MCM10 expression (IHC index ≤4). In addition, MCM10 expression was upregulated in all histopathological

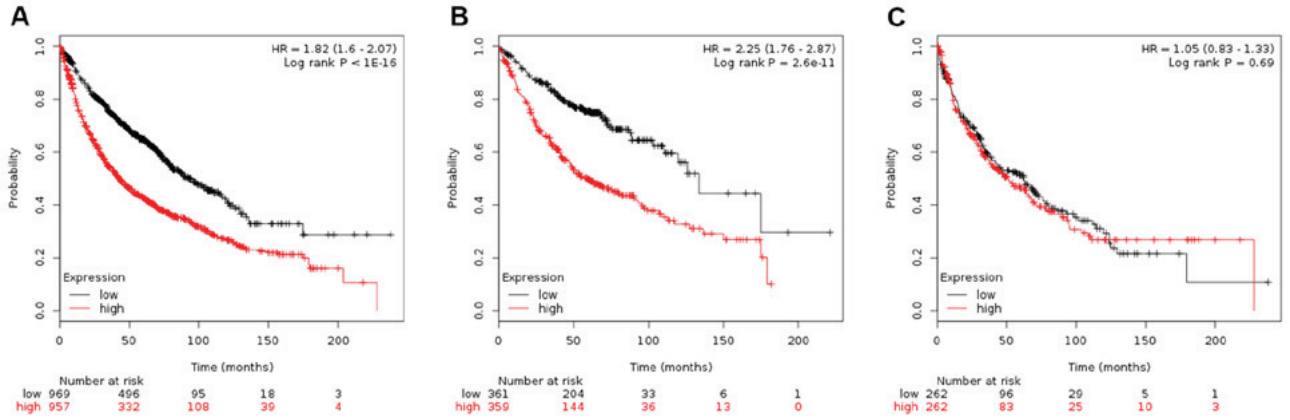

Figure 3. Association between MCM10 expression and survival of patients with lung cancer. Survival curves were plotted for (A) all lung cancer patients (n=1,926), (B) ADC patients (n=720) and (C) SCC patients (n=524). Affymetrix ID: 220651_at (MCM10). Data were analyzed using Kaplan-Meier Plotter. ADC, adenocarcinoma; SCC, squamous cell carcinoma; HR, hazard ratio; MCM10, minichromosome maintenance complex component 10.

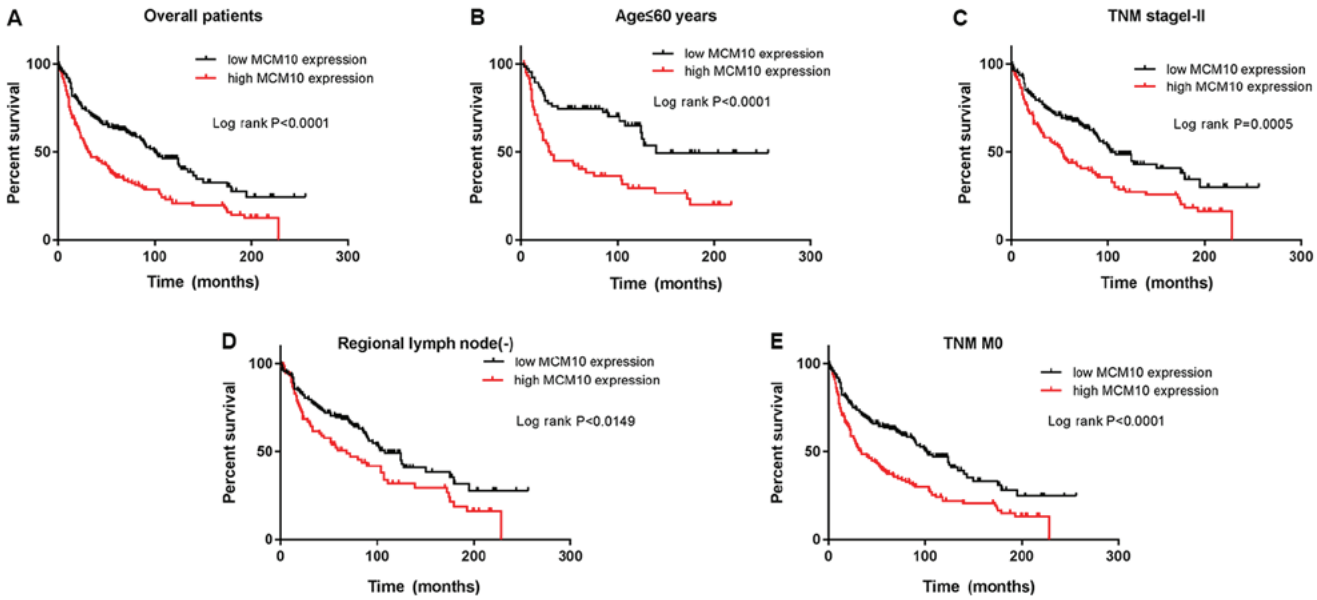

Figure 4. Potential prognostic value of MCM10 expression in lung cancer patients using the GSE30219 dataset. Stratified analysis of the association between MCM10 expression and overall survival of (A) all lung cancer patients, (B) patients aged ≤60 years, (C) patients with stage I-II lung cancer, (D) patients with negative lymph node infiltration, and (E) patients with no distant metastasis. MCM10, minichromosome maintenance complex component 10.

types of lung cancer compared with the adjacent normal tissue (Fig. 2B). These findings suggested an oncogenic potential of MCM10 in the pathogenesis of lung cancer.

Increased expression of MCM10 is associated with more aggressive clinicopathological features of lung cancer. GSE31210 dataset was used to investigate whether MCM10 expression was associated with clinical pathological features of lung cancer. An increased expression level of MCM10 was significantly associated with the pathological properties of lung cancer, such as pathological stage, early recurrence and late recurrence, without having a significant association with age and gender (Table II).

Higher MCM10 expression predicts poor survival in lung cancer patients. The KM plotter database was used to analyze the prognostic potential of MCM10 in lung cancer. The categorization of MCM10 expression (low vs. high) was based on the median expression level. High MCM10 expression level contributed to the shorter OS time of patients with lung cancer [n=1,926; HR, 1.82; 95% CI, 1.6-2.07, log rank P<$1x10^{-16}$; Fig. 3A]. The current study also analyzed the association between MCM10 expression and the prognosis of patients with ADC and SCC. The results revealed that increased MCM10 expression had a negative effect on the survival of ADC patients [n=720; HR 2.25; 95% CI, 1.76-2.87, log rank P=$2.6x10^{-11}$; Fig. 3B]. However, no difference was observed among SCC patients [n=524, HR 1.05; 95% CI, 0.83-1.33), log rank P=0.69, Fig. 3C]. In addition, analysis of the GSE30219 dataset (n=293), revealed a shorter OS time for patient with high MCM10 expression compared with patients with low MCM10 expression (Fig. 4A). The prognostic value of MCM10 was also evaluated with respect to clinicopathological features of lung cancer patients. A positive association between MCM10

Table III. GSEA of the biological process associated with minichromosome maintenance complex component 10 enrichment in lung cancer.

Biological process	ES	NES	P-value	FDR q-value
Double strand break repair	0.826855	2.246242	<0.001	0.002757
DNA metabolic process	0.567782	2.215505	<0.001	0.001379
Cell cycle process	0.626299	2.19163	<0.001	$9.19x10^{-4}$
DNA recombination	0.68314	2.181545	<0.001	0.001528
Cell cycle phase	0.615538	2.177247	<0.001	0.001433
DNA repair	0.588894	2.151542	<0.001	0.001549
Response to DNA damage stimulus	0.560623	2.144127	<0.001	0.001838
DNA dependent DNA replication	0.679776	2.139615	<0.001	0.001608
Mitotic cell cycle	0.632045	2.137272	<0.001	0.00143
Organelle lumen	0.550856	2.132152	<0.001	0.001287
Membrane enclosed lumen	0.550856	2.132152	<0.001	0.00117
DNA replication	0.641062	2.128768	<0.001	0.001072
Cell cycle checkpoint go 0000075	0.674331	2.119582	<0.001	0.001227
Chromosome	0.648138	2.111891	<0.001	0.001221
Cell cycle go 0007049	0.544098	2.110261	<0.001	0.001205
Response to endogenous stimulus	0.53166	2.107898	<0.001	0.001185
Mitochondrion	0.588057	2.104701	<0.001	0.001293
Nuclear part	0.564035	2.102454	<0.001	0.001327
M phase	0.629259	2.097155	0.001961	0.001372
Mitochondrial part	0.663608	2.09199	<0.001	0.001354

GSEA, gene set enrichment analysis; NES, normal enrichment score; FDR q-value, false discovery rate q-value.

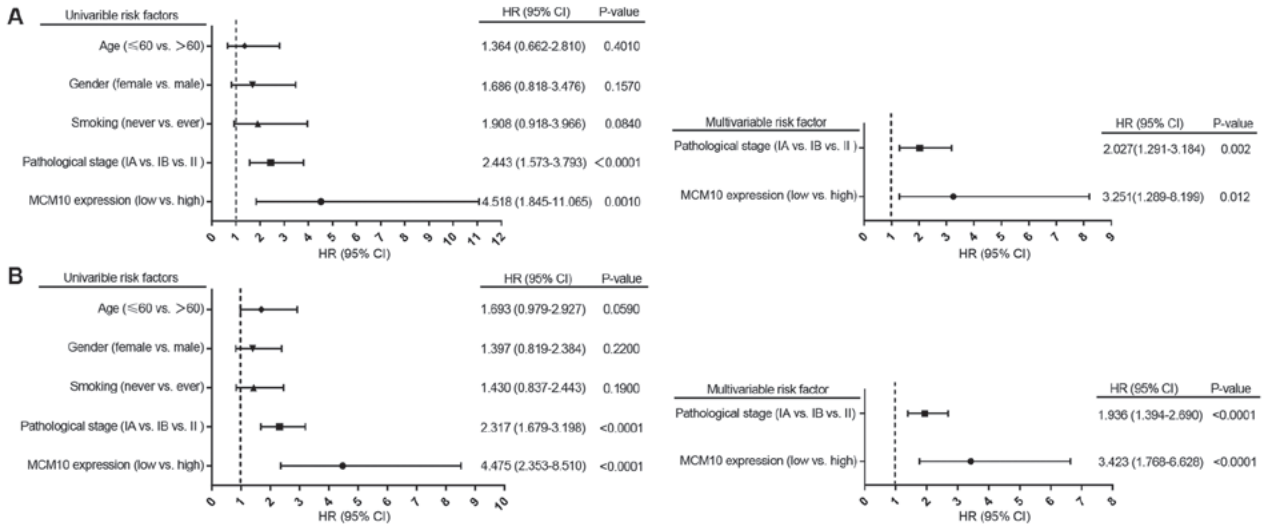

Figure 5. MCM10 expression is an independent risk factor for overall survival and recurrence-free survival. (A) Association between overall survival of lung cancer patients and MCM10 expression as well as other clinical characteristics analyzed using univariate and multivariate analyses. (B) Association between disease-free survival of lung cancer patients and MCM10 expression as well as other clinical characteristics analyzed using univariate and multivariate analyses. HR, hazard ratio; CI, confidence interval; MCM10, minichromosome maintenance complex component 10.

expression and decreased survival probability was observed in lung cancer patients with less malignant characteristics such as age ≤60 years, early T-stage, negative lymph node infiltration and no distant metastasis. The KM analysis indicated a longer median OS for patients with reduced MCM10 expression in the stratified subgroups (all P≤0.0149; Fig. 4B-E).

MCM10 expression is an independent predictor for OS and recurrence-free survival of lung cancer patients. In order to investigate the role of MCM10 expression as an independent predictor of a shorter OS and recurrence-free survival in early stages of lung cancer, the GSE31210 dataset was used for prognosis analysis. This dataset contained 204 cases

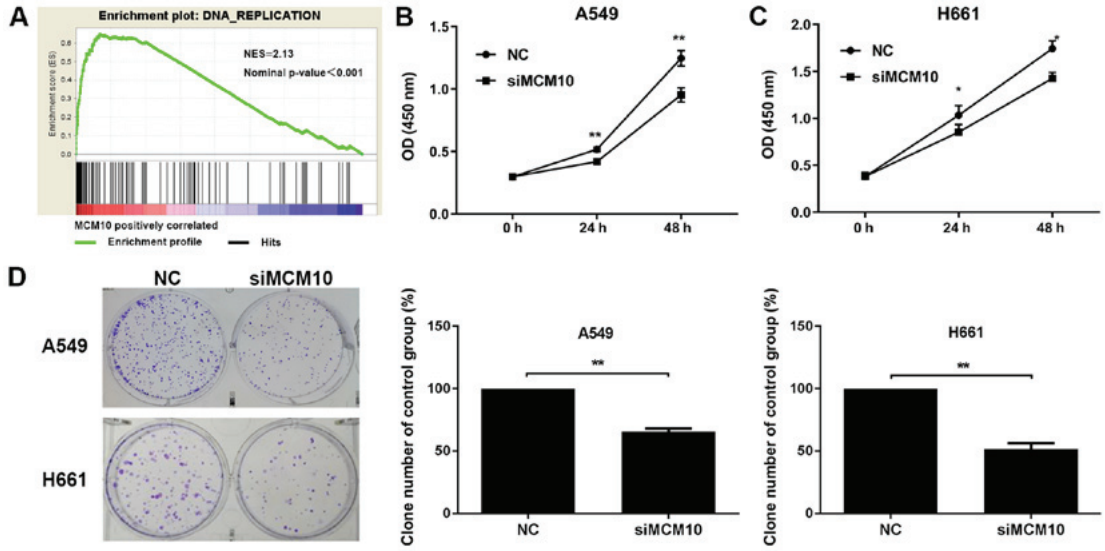

Figure 6. Inhibition of lung cancer cell proliferation by the knockdown of MCM10. (A) Gene set enrichment analysis using the GSE3141 dataset to obtain a gene set associated with high MCM10 expression. Cell Counting Kit-8 assays were performed using (B) A549 and (C) H661 cells to analyze proliferation of NC or siMCM10 groups at the indicated time points. (D) Colony formation assay to reveal the effect of siMCM10 on A549 and H661 cells compared with NC (magnification, x1). Data are presented as the mean ± SEM of three independent experiments, each performed in triplicate. *P<0.05 and **P<0.01 vs. siMCM10. MCM10, minichromosome maintenance complex component 10; NC, negative control; siMCM10, small interfering RNA targeting MCM10; OD, optical density, NES, normal enrichment score.

of I-II stage lung cancer; however, 22 cases were excluded due to incomplete resection or adjuvant therapy. Univariate analysis showed that high MCM10 expression (P=0.001) and pathological stage II (P<0.0001) were associated with poor OS in lung cancer patients (Fig. 5A). The incorporation of the significant characteristics into the multivariate analysis revealed that pathological stage (HR, 2.027; 95% CI, 1.291-3.184; P=0.002) and MCM10 expression (HR, 3.251; 95% CI, 1.289-8.199; P=0.012) were independent prognostic factors of OS. Furthermore, the current results also indicated that MCM10 expression was an independent prognostic factor for recurrence-free survival both in univariate analysis (HR, 4.475; 95% CI, 2.353-8.510; P<0.0001) and multivariate analysis (HR, 3.423; 95% CI, 1.768-6.628; P<0.0001; Fig. 5B).

Inhibition of lung cancer cell proliferation by the knockdown of MCM10. GSEA was performed using the GSE3141 dataset to understand the cellular processes in the pathogenesis of lung cancer mediated by increased expression of MCM10. The samples were divided into two groups (high vs. low) using the median expression level of MCM10, and the top 20 related biological processes that satisfied P<0.05 and false discovery rate <0.25 are shown in Table III. The results suggested that the significant gene set regulated by MCM10 was mainly associated with cell cycle progression and proliferation (Fig. 6A and Table III). To confirm the GSEA analysis of MCM10, MCM10 expression was transiently knocked down in A549 and H661 cells followed by the assessment of the effect of MCM10 expression on cell proliferation using the CCK-8 assay. The results showed that the downregulation of MCM10 significantly suppressed cell proliferation (Fig. 6B and C). Consistent with the findings of the CCK-8 assay, colony formation assay also showed that lung cancer cell growth was significantly inhibited when MCM10 was knocked down (Fig. 6D).

Downregulation of MCM10 induces G1 arrest by suppressing cyclin D1 expression in vitro. Several previous studies demonstrated the function of MCM10 in DNA replication (9,12,26); however, the effect of MCM10 on cell cycle regulation in lung cancer remains to be elucidated. Flow cytometry analysis was used to determine the effect of MCM10 knockdown on lung cancer cell cycle progression. As shown in Fig. 7A, the depletion of MCM10 significantly increased and decreased the proportion of G_0/G_1 and G_2/M phase cells, respectively compared with the NC group in A549 cells, while knockdown MCM10 only caused G_0/G_1 arrest in H661 cells. An apoptosis assay was performed after 48 h of transfection; however, no significant differences were observed between the two groups (Fig. S3). To explore the mechanism of proliferation inhibition induced by MCM10 knockdown, RT-qPCR was performed to screen a spectrum of genes related to cell cycle regulation between NC and MCM10 knockdown groups. CCND1, a gene responsible for G_1/S transition, was decreased to the greatest extent at the mRNA level following siMCM10 transfection (Fig. 7B and C). To confirm the effect of MCM10 on CCND1 expression at the protein level, proteins were extracted from A549 and H661 cells transfected with siMCM10 or NC. Western blotting results revealed that CCND1 was expressed at a low level in siMCM10 cells compared with the NC group (Fig. 7D).

MCM10 and CCND1 can serve as prognostic indicators of lung cancer. The potential value of CCND1 expression in predicting the survival of patients with different pathological subtypes of lung cancer was determined in the current study (Fig. S4). The results showed that high CCND1 expression only predicted a worse outcome in patients with stage I-II and negative regional lymph node infiltration compared with low CCND1 expression. Due to the aforementioned association between MCM10 and CCND1, the current study explored the combination of MCM10 and CCND1 in the prognosis predic-

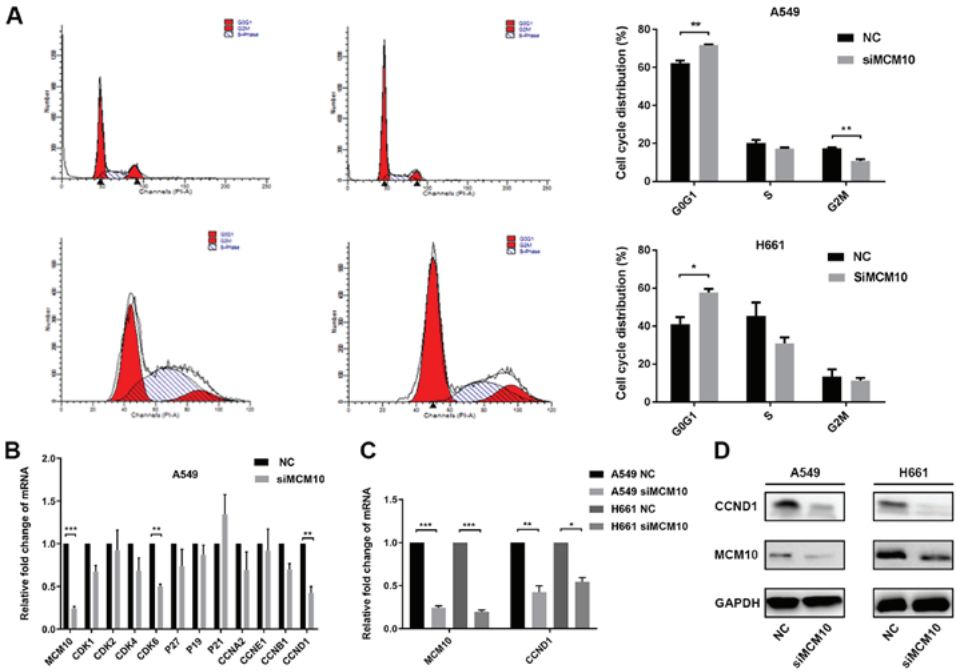

Figure 7. Downregulation of MCM10 induces G1 arrest by suppressing CCND1 expression *in vitro*. (A) Flow cytometry analysis of cell-cycle in A549 and H661 cells with or without MCM10 depletion. (B) Reverse transcription-quantitative PCR was performed to explore the expression of CDK1, CDK2, CDK4, CDK6, P27, P19, P21, CCNA2, CCND1, CCNB1, CCNE1 in siMCM10 and NC groups of A549 cells. (C) Relative expression of CCND1 after MCM10 knockdown in A549 and H661 cells. (D) Western blotting assay showing MCM10 and CCND1 protein expression level after MCM10 knockdown in A549 and H661 cells. GAPDH was used as a loading control. Data are presented as the mean ± SEM of three independent experiments, each performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001. CCND1, cyclin D1; CDK, cyclin-dependent kinase; CCNA2, cyclin A2; CCNB1, cyclin B1; CCNE1, cyclin E1; MCM10, minichromosome maintenance complex component 10; NC, negative control; siMCM10, small interfering RNA targeting MCM10.

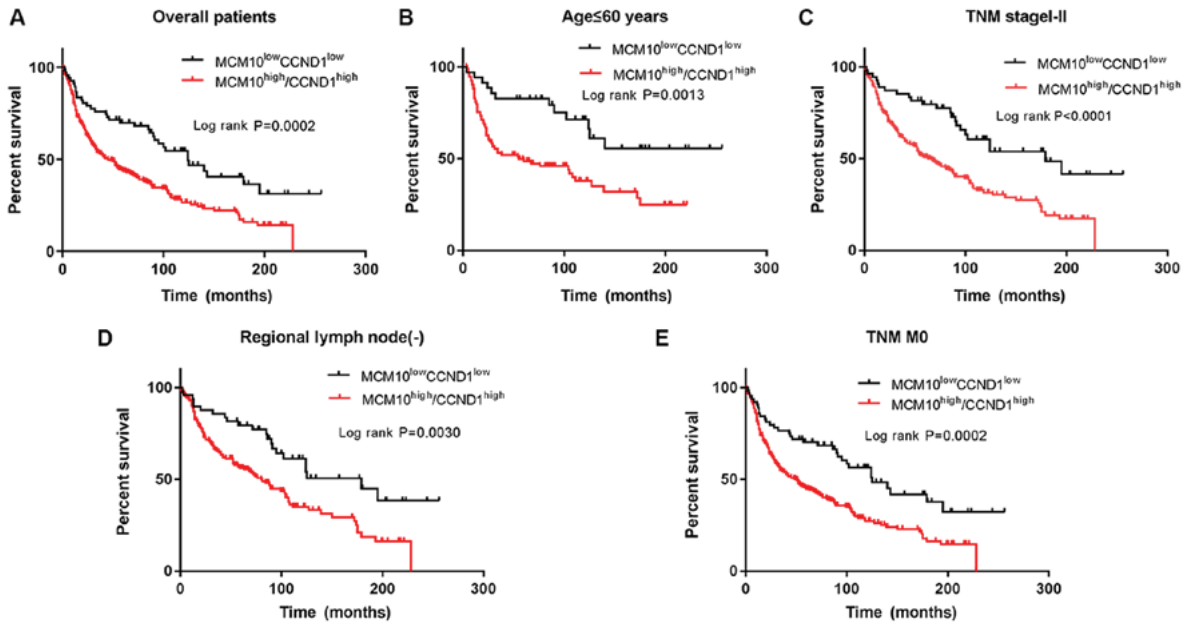

Figure 8. MCM10 and CCND1 can serve as prognostic indicators of lung cancer. Prognosis in (A) all lung cancer patients, (B) patients aged ≤60 years, (C) patients with stage I-II lung cancer, (D) patients with negative lymph node infiltration, and (E) patients with no distant metastasis. MCM10, minichromosome maintenance complex component 10; CCND1, cyclin D1.

tion of patients with lung cancer. Patients were divided into an MCM10 and CCND1 low expression group and an MCM10 and CCND1 high expression group to obtain KM plots based on the GSE30219 dataset. Combination of low MCM10 and CCND1 expression was associated with a longer median OS (Fig. 8A). In addition, low MCM10 and CCND1 expression was associated with better outcomes compared with high MCM10 and CCND1 expression in patients with less aggressive characteristics such as age ≤60 years, early T-phase, negative lymph node infiltration and no distant metastasis (Fig. 8B-E),

demonstrating that the combination of MCM10 and CCND1 expression may be a prognostic indicator for early-stage lung cancer patients.

Discussion

Aberrant DNA replication is a hallmark of cancer (27). Certain molecules associated with DNA replication are abnormally highly expressed in proliferating cells, especially in a variety of tumor cells (28-31). In the current study, existing clinical samples were analyzed to confirm the increased expression of MCM10 in lung cancer. It was also revealed that MCM10 overexpression was associated with more aggressive clinicopathological characteristics of lung cancer. Further analysis revealed that high expression of MCM10 predicted worse OS of patients with stage I cancer with no regional lymph node infiltration or distant metastasis suggesting that MCM10 combined with traditional TNM classification may provide more precise criteria for prognostic judgment. In addition, overexpression of MCMs in early stages of cell cycle has been documented by a number of studies, and may be ideal tumor marker for the early stage of proliferation (32-34). The current study, to the best of our knowledge, is the first to report an increased level of MCM10 expression in the IA stage of lung cancer demonstrating a possible application of MCM10 as a biomarker in the screening for early stages of lung cancer.

The precise role of MCM10 upregulation in lung cancer is not fully elucidated. Previous studies demonstrated an increased expression of MCM10 during the G_1/S transition and a decreased expression level of MCM10 in the G_2/M phase of the cell cycle (35-37). MCM10 gene silencing in HeLa cells led to inhibition of DNA synthesis thereby indicating that depletion of MCM10 expression may induce replication process aberrations (38,39). The current study suggested that knockdown of MCM10 resulted in cell growth inhibition and G0/G1 arrest.

To elucidate the mechanisms underlying MCM10-mediated cell proliferation, several studies have found upstream and downstream factors that could activate MCM10 (40-43). The E2F/retinoblastoma tumor suppressor protein pathway, which serves an important role in cell cycle and proliferation control, induced the expression of MCM10 in HCT116 cells (40). MicroRNAs (miRs) such as miR-146-5p have been found to regulate the expression level of MCM10 (41), and depletion of sirtuin 1 by siRNA resulted in increased levels of MCM10 and chromatin binding activity (42). Furthermore, in neuroblastoma, a strong positive correlation was observed between MCM10 and N-MYC which caused the upregulation of MCM10 by binding to its promoter (43). Similarly, a previous study investigated the regulation of MCM10 by in Ewing's sarcoma by EWS/friend leukemia integration 1 transcription factor and nuclear receptor subfamily 0 group B member 1 (17,43). The current study revealed that the expression of CCND1 was significantly inhibited in lung cancer cell lines after MCM10 downregulation causing G_1 arrest of cell cycle, indicating that CCND1 may be a downstream target of MCM10. CCND1 is a cell cycle-related gene involved in tumorigenesis (44). The combination of MCM0 and CCND1 in the prognosis prediction of patients with lung cancer was therefore further predicted., the difference between low expression of MCM10 and CCND1 and high expression of MCM10 and CCND1 was significant. The result suggested that the combination of MCM0 and CCND1 may be a prognostic indicator for relatively early-stage lung cancer patients.

In conclusion, the role of MCM10 was explored by validating its oncogenic and prognostic potential in lung cancer. High MCM10 was associated with poor prognosis in lung cancer patient. Moreover, MCM10 was found to promote lung cancer cell proliferation via upregulating CCND1. The current findings suggested the potential application of MCM10 as a biomarker for the prognosis of lung cancer.

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Availability of data and materials

The GEO datasets are available on National Center for Biotechnology Information (National Institutes of Health; http://www.ncbi.nlm.nih.gov/geo/). The online database Kaplan Meier Plotter can be accessed on http://kmplot.com/analysis/index.php?p=service&cancer=lung. The Oncomine data are available on https://www.oncomine.org. The expression level of MCM10 in the Cancer Genome Atlas dataset was determined using UALCAN (http://ualcan.path.uab.edu). The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MW, SX and QZ conceived and designed the study. MW, SX and WY conducted the experiments. TX and JH were involved in statistical analysis. QY collected clinical samples and interpreted the experimental results. MJ and HS wrote and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China). Written informed consent was obtained from all patients.

Patient consent for publication

Written informed consent was obtained from all examined patients for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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